Phosphate and the Development of Nephrocalcinosis in Rats Fed Diets Containing Alpha Protein

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It has been suggested that nephrocalcinosis in rats fed diets containing alkali-treated soy protein may be due to a high availability of phosphate in the diet. In the present study, the development of nephrocalcinosis in rats fed a diet containing 20% alpha protein (an alkali-treated soy protein) was compared with that in rats fed the same diet supplemented with additional phosphate. Phosphate supplementation of the alpha protein diet produced a form of nephrocalcinosis that was morphologically different, at both the light- and electron-microscopic level, from that obtained with the unsupplemented diet but was quite similar to that obtained with a phosphate-supplemented standard commercial laboratory diet. Levels of serum and urinary calcium and phosphorus and urinary cyclic AMP suggested that a phosphate-induced secondary hyperparathyroidism was present in the rats fed either of the phosphate-supplemented diets, but not in the rats fed the unsupplemented alpha protein diet. The results of this study suggest that nephrocalcinosis in rats fed a diet containing 20% alpha protein, without additional phosphate, is not typically phosphate-induced (Am J Pathol 1983, 113:95-106).

SINCE De Groot and Slump1 and Woodard2 first described nephrocalcinosis in rats fed diets containing alkali-treated soy protein, the cause of the calcification has never been fully determined. Woodard3 ruled out the effect of a “toxin” produced during the alkali treatment when he found the same type of nephrocalcinosis in rats fed diets containing soy protein that was not treated with alkali. Others,1,4,5 however, maintained that the calcification was more severe with alkali-treated soy protein.

As a result of varying the composition of the soy protein diet, Woodard6 determined that an interaction between mineral and nonmineral components was responsible for the calcification. Kaunitz and Johnson7 thought that the development of nephrocalcinosis was more dependent on the type of protein in the diet. They found a more severe form of nephrocalcinosis in rats fed diets containing soy protein or casein than in rats fed a diet containing lactalbumin, even though the mineral contents of all three diets were similar. Like Woodard, Van Beek et al4 felt that minerals and, in particular, phosphate played a major role in the calcification process. Because nephrocalcinosis was more severe in rats fed diets containing alkali-treated soy protein than in rats fed diets containing an untreated soy protein, Van Beek et al suggested that the phosphate in the alkali-treated soy protein diets was somehow “more available.” In addition, attention was drawn to the similarities between nephrocalcinosis induced by soy protein diets and that induced in rats fed special, high-phosphate diets.8 We therefore carried out the present study to examine more closely the effects of phosphate on the development of nephrocalcinosis in rats fed a diet containing alkali-treated soy protein.

Materials and Methods

Female, weanling Sprague–Dawley rats weighing 40–60 g were used in all the experiments. The study was carried out in two phases.

Phase 1

We performed preliminary experiments to trace the development of nephrocalcinosis in rats fed a diet

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Table 1—Composition of the Basal Alpha Protein and Phosphate-Supplemented Alpha Protein and Commercial Laboratory Diets*

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<th>Ingredient</th>
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<th>High phosphate</th>
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<td>Alphacet†</td>
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<td>2.5</td>
</tr>
<tr>
<td>Dextrin†</td>
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</table>

* The phosphate-supplemented commercial laboratory diet was made up by adding 65.4 g potassium dihydrogen phosphate (KH₂PO₄) to every 1 kg of the basic diet. (Raleton Purina of Canada Ltd., Woodstock, Ontario, Canada).
† I.C.N. Nutritional Biochemicals, Montreal, Quebec, Canada, HAM 1V1.
‡ Salt Mix Composition (g/100 g mix). Basal diet: CaHPO₄·2H₂O 43.33, CaCO₃ 7.69, NaCl 3.81, KCl 1.88, MgO 1.99, Fe citrate 2.99, ZnCO₃ 0.43, MnSO₄·H₂O 0.32, CuSO₄·5H₂O 0.24, KIO₃ 0.0055. High-phosphate diet: CaH₂PO₄·H₂O 82.64, KH₂PO₄ 13.17, NaCl 3.81, KCl 0.82, MgO 1.99, Fe citrate 2.99, ZnCO₃ 0.43, MnSO₄·H₂O 0.32, CuSO₄·5H₂O 0.24, KIO₃ 0.0055.
§ Vitamin mix composition (g/100 g mix): thiamine HCl 0.32, pyridoxine HCl 0.32, riboflavin 0.32, δ-calcium pantothenate 0.8, nicotinamide 1.0, folic acid 0.1, inositol 2.0, alpha-ol-tocopherol acetate 0.7, menadione 0.004, vitamin B₆ 0.02, biotin 0.002, vitamin A acetate (500,000 IU/g) 0.001, vitamin D₃ (500,000 IU/g) 0.024.

containing 20% alpha protein. The diet, modeled after that of Woodard and Short⁶ (Table 1), was fed to groups of 4 rats for 3, 6, 9, and 12 weeks. Control groups, also consisting of 4 animals each, were fed a standard commercial laboratory diet for corresponding periods. The ratio of calcium to phosphorus of both diets was approximately 1:0.7.

Phase 2

The second part of the study dealt with the effects of increasing the level of phosphate in the diet on the development of nephrocalcinosis. Groups of 4 rats were fed the 20% alpha protein diet or the commercial laboratory diet, to which was added inorganic phosphate (Table 1), for 3 days and 1, 3, and 6 weeks. The calcium/phosphorus ratio of both phosphate-supplemented diets was approximately 1:2. Corresponding control groups, also consisting of 4 animals each, were fed the unsupplemented diets.

During the experiments all rats were housed individually in stainless steel metabolic cages and were allowed food and water ad libitum. At least once a week, each animal was weighed, and 24-hour food consumption measurement was made. Periodic blood and 24-hour urine samples were collected for calcium and phosphorus and cyclic AMP (urinary) determinations, except in the preliminary experiments. The blood samples were taken from the orbital sinus under ether anesthesia, and urine collections were made in dry ice and kept frozen until the biochemical determinations were made.

At the end of the experiments the rats were anesthetized with ether. The left kidney was removed and sliced transversely. One slice was fixed in 80% ethanol for 24 hours at 5 °C and then for a further 24 hours at room temperature, and another slice was fixed in neutral buffered formalin for 48 hours at room temperature. After fixation, the slices were dehydrated in a graded series of ethanol, cleared in toluene, and embedded in paraffin wax for light microscopy. Sections, 6–10 μ thick, of the ethanol-fixed tissue were stained with Alizarin red S⁸ or by the combined von Kossa–PAS method¹¹ for demonstration of the presence of calcium salts. Sections, 5 μ thick, of the formalin-fixed tissue were stained with hematoxylin and eosin (H&E) for general histologic study.

An additional slice from some kidneys was taken and processed for transmission electron microscopy. Each slice was quickly immersed in fixative containing 2.5% glutaraldehyde, 2.0% paraformaldehyde, buffered with 0.1 M s-collidine (pH 7.4). Samples from the cortex, the inner and outer stripes of the outer medulla, and the inner medulla were diced into small cubes (1 cu mm) and fixed for a further 4–6 hours. The samples were then postfixed for 1 hour in 1% osmium tetroxide, buffered with 0.1 M s-collidine. Subsequently, the samples were stained en bloc for 1 hour with 2% uranyl acetate. After staining, the specimens were dehydrated in a series of graded ethanols and washed in propylene oxide. The blocks were then infiltrated with a half-and-half mixture of propylene oxide and Araldite CY212 for 4 hours or overnight, followed by pure Araldite for 4–6 hours. Finally, the tissue blocks were embedded in Araldite at 60 °C for 24 hours.

The right kidneys were either processed for light microscopy, as described above, or dehydrated in an incubator at 80 °C for 2 weeks. After dehydration, the kidneys were immersed in petroleum ether for 4–6 hours for the removal of fat. The fat-free dry kidneys were then ashed in a Sybron Thermolyne 1500 muffle furnace at 600 °C for 48 hours. The resulting residue from each kidney was dissolved in three 1-ml aliquots of 1 N HCl for the determinations of total renal calcium and phosphorus.

All phosphorus determinations were made with the use of the Phosphorus Auto/Stat Kit (Pierce Chemical Co., Rockford, Ill) spectrophotometric method. Calcium measurements were made with the use of the
Calcium Rapid Stat Kit (Pierce Chemical Co.) spectrophotometric method or a Varian atomic absorption spectrophotometer. Urinary cyclic adenosine monophosphate (AMP) was measured using a cyclic AMP Assay Kit (Amersham Corporation, Oakville, Ontario, Canada) and an LKB 1215 Rackbeta liquid scintillation counter.

Statistical assessments of the differences between means for each set of quantitative data were carried out with the use of one-way analysis of variance (preliminary experiments) or two-way analysis of variance, followed by multiple comparison testing with the use of the least significant difference method.12

Results

Preliminary Experiments

In none of the experiments was there a difference between experimental and control groups with respect to weight gain and food consumption.

Light Microscopy

Animals fed the basic alpha protein diet for 3 weeks developed a mild form of nephrocalcinosis. A few deposits, staining with both Alizarin red S and by the von Kossa technique, were found in the inner medulla. Otherwise, the kidneys appeared normal. Although the degree of calcification did not increase significantly after 6 weeks, there was a difference in the distribution of deposits. In addition to some deposits in the inner medulla, others were in the lumens of tubules situated at the junction of the outer and inner stripes of the outer medulla and in the inner stripe of the outer medulla. The deposits appeared to be localized primarily in pars recta segments of proximal tubules and in descending thin limbs of Henle. Some epithelial cell necrosis was detected in the proximal tubules, but only in proximity to the calciferous deposits. No other changes were detected after 6 weeks of feeding.

After 9 weeks, the severity of nephrocalcinosis increased in the rats fed the alpha protein diet. Most of the calciferous deposits were found in the lumens of proximal tubules situated at the junction of the outer and inner stripes of the outer medulla, where a distinct band of calcification was visible (Figures 1 and 2). Some deposits were also found in descending thin limbs of Henle. Proximal tubular necrosis was more prominent than at 6 weeks but again was detected only near the calciferous deposits. In the animals fed the alpha protein diet for 12 weeks the characteristics of nephrocalcinosis were the same as those found after 9 weeks.

No calcification or other pathologic changes were detected in the kidneys from control rats fed the commercial laboratory diet.

Electron Microscopy

Ultrastructural changes in the kidneys from rats fed the alpha protein diet for 12 weeks supported the light-microscopic findings. Intraluminal deposits and necrotic epithelial cells, particularly in the terminal segments of proximal tubules, were the most frequent findings. Some of the necrosis was very severe around a number of deposits, which made it impossible to identify any normal features of proximal tubular cells. The deposits ranged in size from small lamellar bodies (Figure 3) to structures that completely filled the tubular lumen. Most of the larger deposits were fragmented during sectioning, and therefore it was difficult to characterize them as well as get examples for electron micrography, but it appeared that a number of deposits consisted of concentric lamellas wrapped around a central core (Figure 4). It seemed that many of the deposits may have been formed in part by or from proximal tubular cells inasmuch as they were in close approximation with portions of necrotic epithelial cells of proximal tubules. Occasional apatitelike crystals were found within some of the epithelial cells and between the microvilli of the brush border of proximal tubules (Figure 5). Some degeneration and breakdown of the microvilli were also apparent.

Lamellar bodies were also found in the lumens of some descending thin limbs of Henle (Figure 6). No obvious changes were seen in the epithelial cells surrounding the deposits.

Quantitative Results

Total renal calcium and phosphorus values were significantly greater in the rats fed the alpha protein diet for 12 weeks than in the corresponding control rats fed the commercial laboratory diet (Table 2), thus supporting the morphologic findings demonstrating the presence of nephrocalcinosis.

Phosphate-Supplemented Experiments

As in the preliminary experiments, there was no difference between experimental and control groups with respect to weight gain and food consumption.

Light Microscopy

Nephrocalcinosis developed more rapidly and was more severe in rats fed the phosphate-supplemented diets than in the rats fed the basal alpha protein diet in the preliminary experiments. The localization of
Figure 1—Kidney from a rat fed a diet containing 20% alpha protein for 9 weeks. Intraluminal calciferous deposits (black) are present at the junction of the outer and inner stripes of the outer medulla (OS/IS). Some appear to be in the terminal segments of proximal tubules, which can be identified by their brush border (BB). (von Kossa–PAS, ×30)

Figure 2—A calciferous deposit in a terminal segment of a proximal tubule (PT) in the kidney of a rat fed a diet containing 20% alpha protein for 9 weeks. (H&E, ×700)

Figure 3—Lamellar bodies (LB) and a calcified mass (CM) in the lumen of a severely damaged tubule at the junction of the outer and inner stripes of the outer medulla. Kidney from a rat fed a diet containing 20% alpha protein for 12 weeks. (×4000)

Figure 4—An intraluminal, lamellar deposit at the junction of the outer and inner stripes of the outer medulla of a kidney from a rat fed a diet containing 20% alpha protein for 12 weeks. Concentric lamelllas are seen in the outer portion of the deposit in the calcified tubule (CT). Only fragments of a central electron-dense core are present. (×3300)

Figure 5—Calcified brush border (CBB) of a proximal tubule in the kidney of a rat fed a diet containing 20% alpha protein for 12 weeks. Apatiticlike crystals appear to be in between and around the microvilli. (×8000)

Figure 6—Lamellar body (large arrow) and electron-dense membranous debris (small arrows) in the lumen (L) of a descending thin limb (TL) of Henle. Note that the epithelial cells of the tubule appear relatively normal. Kidney from a rat fed a diet containing 20% alpha protein for 12 weeks. (×4700)
the early deposits was also different. In rats fed the phosphate-supplemented alpha protein diet for 3 days large intraluminal deposits were present in the inner stripe of the outer medulla, specifically in the collecting ducts and the ascending thick limbs of Henle. However, some deposits were also found in descending thin limbs of Henle. This was in addition to numerous small deposits in the lumens of collecting ducts situated in the inner medulla. Only occasional, small deposits were seen at the junction of the outer and inner stripes of the outer medulla, and these were in terminal segments of proximal tubules.

A similar pattern of calcification was found in the group fed the phosphate-supplemented commercial laboratory diet for 3 days, but the number of deposits in each of the regions of the kidney was much lower.

In animals fed the phosphate-supplemented alpha protein diet for 1 week the histologic findings were essentially the same as those seen after 3 days (Figure 7). However, in the group fed the phosphate-supplemented commercial laboratory diet the nephrocalcinosis was much more pronounced (Figure 8). It appeared as if two distinct bands of calcification were forming, one at the junction of the outer and inner stripes of the outer medulla and the other more prominent band in the inner stripe of the outer medulla. In both groups fed the phosphate-supplemented diets degeneration, necrosis, and calcification of epithelial cells surrounding the deposits were apparent. In addition, inflammatory cells were present in the peritubular spaces around the more heavily calcified tubules. Some foreign-body giant cells were also found in the inner stripe of the outer medulla.

After 3 weeks, renal calcification was more extensive. In the group fed the phosphate-supplemented alpha protein diet the numbers of affected tubules in the inner stripe and at the junction of the outer and inner stripes of the outer medulla were greater. Some deposits were also found in the cortex.

In the more heavily calcified kidneys of rats fed the phosphate-supplemented commercial laboratory diet, the bands of calcification at the junction of the outer and inner stripes and in the inner stripe of the outer medulla were again apparent. Some of the affected tubules were greatly dilated, and deposits were found in the cortex.

Severe tubular damage was evident in both groups fed the phosphate-supplemented diets after 3 weeks (Figures 9 and 10). In addition, many inflammatory cells were found in the interstitial and peritubular spaces around the more heavily calcified tubules in the inner stripe of the outer medulla, as well as, in some instances, around groups of tubules in the inner stripe that were minimally affected by calcification (Figure 11). Some calcified tubules were surrounded by cells resembling fibroblasts, suggesting some attempt at tubular repair. There was also an increased number of foreign-body giant cells in the inner stripe of the outer medulla.

There was a further increase in the severity of nephrocalcinosis after 6 weeks in both groups fed the phosphate-supplemented diets (Figures 12 and 13), but the distribution of deposits was similar to that found after 3 weeks. Although the calcification was more pronounced in the group fed the phosphate-supplemented commercial laboratory diet, there was marked tubular dilation and necrosis in the renal cortex and medulla of both groups. There was also evidence of tubular (Figure 14) and interstitial fibrosis (Figure 15), inflammation, and foreign-body giant cell reaction.

The kidneys of most of the control groups appeared normal. Occasional intraluminal calciferous deposits were found, however, in the inner medulla of the kidneys from rats fed the unsupplemented alpha protein diet for 3 weeks or at the junction of the outer and inner stripes and in the inner stripe of the outer medulla of the kidneys from rats fed the same diet for 6 weeks. These findings confirm the histologic findings of the preliminary experiments.

**Electron Microscopy**

A number of ultrastructural changes were found in the kidneys of rats fed the phosphate-supplemented diets. The changes were essentially the same in both groups, and some were found in each of the 3-day, 1-week, 3-week, and 6-week series.

Large lamellar, intraluminal deposits (5–50 μ in diameter), similar to those seen in the kidneys of rats fed the unsupplemented alpha protein diet for 12 weeks, were found, mainly in pars recta segments of
Figure 7—Transverse section of a kidney from a rat fed a phosphate-supplemented alpha protein diet for 1 week. Many calciferous deposits (black) are situated in the inner stripe of the outer medulla (IS), where a band of calcification appears to have formed. Some deposits are also seen in the inner medulla (right side of photomicrograph). (Alizarin red S, ×45)

Figure 8—Transverse section of a kidney from a rat fed a phosphate-supplemented commercial laboratory diet for 1 week. Calcification appears to be more severe than that seen in Figure 7. Most of the calciferous deposits are situated in the inner stripe of the outer medulla (IS). Some deposits are also seen at the junction of the outer and inner stripes of the outer medulla (left side of photomicrograph). (Alizarin red S, ×45)

Figure 9—Severe tubular damage in the inner stripe of the outer medulla of a kidney from a rat fed a phosphate-supplemented alpha protein diet for 3 weeks. A foreign-body giant cell can be seen in the center of the left margin of the photomicrograph. (H&E, ×150)

Figure 10—Severe tubular damage in the inner stripe of the outer medulla of a kidney from a rat fed a phosphate-supplemented commercial laboratory diet for 3 weeks. Note the dark staining deposits in the lumens of some tubules. (H&E, ×230)

Figure 11—Evidence of inflammation in the inner stripe of the outer medulla of a kidney from a rat fed a phosphate-supplemented commercial laboratory diet for 3 weeks. Many nuclei of inflammatory cells can be seen in the peritubular capillaries and interstitial spaces around the tubules. (H&E, ×230)
Apatitelike crystals were observed in epithelial cells and basal laminas of all different types of tubules present in the zones where calcification was most pronounced, especially in the inner stripe of the outer medulla. In some instances where intracellular calcification was quite marked, large numbers of the apatitelike crystals were found to be concentrated within nuclei and around apical, lateral, and basal plasma membranes, while fewer numbers were found in mitochondria and around other membrane-bound structures (Figure 16).

Electron-dense plasma membranes (Figures 17 and 18) were also observed in the epithelial cells of all the various types of tubules present in the zones where calcification was most pronounced. In many cases they were seen in the same cells containing apatitelike crystals. These electron-dense membranes were not found in the rats fed the unsupplemented alpha protein diet for 12 weeks.

Another unique type of deposit, found only in the kidneys of rats fed the phosphate-supplemented diets, was observed in regions normally occupied by the basal laminae of collecting ducts, mainly those segments in the inner stripe of the outer medulla. Cross-sections of the deposits revealed that they consisted of various layers of laminae (50 nm to 1.0 μ in thickness) (Figure 19) and therefore were termed "laminated deposits." In some cases, a laminated deposit was found completely around the basal region of a collecting duct.

In regions where the calciferous deposits were most numerous, degeneration and necrosis of tubular epithelial cells were common. Such cells contained debris-filled intracytoplasmic vacuoles and pyknotic, karyolytic, or karyorrhectic nuclei. Some of these cells appeared to become incorporated into the intraluminal deposits. Occasional mitotic figures were found in the epithelium of proximal tubules and collecting ducts, indicating some attempt at tubular regeneration. Monocytes, lymphocytes, and neutrophils were present in the interstitial spaces and peritubular capillaries around severely damaged tubules. A number of eosinophils were also seen, some of which had migrated between the epithelial cells of degenerating tubules. Large numbers of fibroblasts and collagen fibers were observed in some interstitial spaces, in some instances completely encircling isolated groups of severely calcified tubules.

The ultrastructure of the kidneys of all the control groups appeared normal, with the exception of some epithelial necrosis and a few small intraluminal deposits in the terminal segments of proximal tubules of the kidneys from rats fed the unsupplemented alpha protein diet for 6 weeks.
Figure 14—Fibrosis of a calcified renal cortical tubule. Arrowheads point to cells resembling fibroblasts, which appear to encircle the tubule. Kidney of a rat fed a phosphate-supplemented alpha protein diet for 6 weeks. (H&E, x350)

Figure 15—Interstitial fibrosis in the inner stripe of the outer medulla of a kidney from a rat fed a phosphate-supplemented commercial laboratory diet for 6 weeks. Fibrotic tissue and cells resembling fibroblasts can be seen in the interstitial spaces around the severely damaged tubules. (H&E, x250)

Figure 16—Calcified epithelial cell of a distal tubule in the inner stripe of the outer medulla of a kidney from a rat fed a phosphate-supplemented commercial laboratory diet for 6 weeks. Apatite-like crystals are seen in contact with or closely associated with electron-dense lateral and basal cell membranes (arrows) and throughout the cytoplasm. Note the mass of apatite-like crystals in the nucleus (N). (x 15,000)

Figure 17—Electron-dense plasma membranes of tubules in the inner stripe of the outer medulla. Electron-dense lateral cell membranes (arrowheads) of a distal
Quantitative Results

Both renal calcium and phosphorus were significantly higher in the groups fed the phosphate-supplemented diets, compared with respective control groups (Tables 3 and 4). After 1, 3, and 6 weeks there was significantly more calcium and phosphorus in the kidneys of the group fed the phosphate-supplemented commercial laboratory diet than in the group fed the phosphate-supplemented alpha protein diet.

Serum calcium was lower and serum phosphorus higher in the groups fed the phosphate-supplemented diets than in corresponding control groups. In addition, serum calcium was lower and serum phosphorus higher in the groups fed the phosphate-supplemented commercial laboratory diet than in the groups fed the phosphate-supplemented alpha protein diet.

In the groups fed the phosphate-supplemented diets the urinary excretion of calcium was lower and the urinary excretion of phosphorus higher, compared with respective control groups. After 3 weeks cyclic AMP excretion was also greater in the groups fed the phosphate-supplemented diets, with the greatest effect found in the groups fed the phosphate-supplemented commercial laboratory diet.

No meaningful differences were found in the parameters measured between the groups fed the unsupplemented alpha protein diet and the corresponding groups fed the unsupplemented commercial laboratory diet.

Discussion

The morphologic results of feeding rats a diet containing 20% alpha protein for up to 12 weeks were similar to, though less severe than, those obtained by Woodard and Alvarez13 and Woodard.1 Intrarenal calcification was present mainly at the junction of the outer and inner stripes of the outer medulla. There was no evidence, however, of the enlarged proximal

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**Table 3—Total Renal, Serum, and Urinary Calcium and Phosphorus (Pi) and Urinary Cyclic AMP in Rats Fed the Basal and Phosphate-Supplemented Alpha Protein and Commercial Laboratory Diets for 3 Days to 6 Weeks**

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<th>Serum Pi (mg/dL, mean ± SD)</th>
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<td>10.7 ± 0.2</td>
<td>7.2 ± 0.4</td>
<td>7.9 ± 2.4</td>
<td>46.3 ± 8.6</td>
<td>14.4 ± 4.5</td>
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<td>10.6 ± 0.2</td>
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<td>13.8 ± 0.7</td>
<td>10.0 ± 0.4</td>
<td>9.5 ± 1.3</td>
<td>3.3 ± 2.7</td>
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<td>50.0 ± 17.8</td>
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<td>50.0 ± 17.8</td>
</tr>
<tr>
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<td>5.5 ± 1.6</td>
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* Estimation based on fat-free dry kidney weight.
Table 4—Statistical Comparisons of Means of Quantitative Data from Rats Fed the Basal and Phosphate-Supplemented Alpha Protein and Commercial Laboratory Diets for 3 Days to 6 Weeks

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<th>Renal Pi</th>
<th>Serum calcium</th>
<th>Serum Pi</th>
<th>Urinary calcium</th>
<th>Urinary Pi</th>
<th>Urinary cyclic AMP</th>
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Blank spaces indicate no significant difference.
Group comparisons made; P < 0.05.

tubular cells with large nuclei, which were described by Woodard as nephrocytomegalic cells. In this respect the results of the present study resemble those of De Groot and Slump15 and Van Beek et al,16 who also observed nephrocalcinosis in the absence of nephrocytomegaly after feeding rats a diet containing alkali-treated soy protein. Van Beek et al suggested that the phosphate in the soy protein diets was somehow "more available" to the animals. In other words, the nephrocalcinosis may have been phosphate-induced. If correct, this would be a significant finding, because their diet and the basal alpha protein diet used in this study contained amounts of phosphate within the range recommended by the American Institute of Nutrition14 and the National Research Council15 for normal growth and development in the rat. In the classic studies of nephrocalcinosis induced by excess dietary phosphate,5-16 however, the amounts of phosphate used to bring about renal calcification were much higher. The amounts of phosphate present in the phosphate-supplemented diets in this study were also greatly in excess of the upper recommended limit. Therefore, the nephrocalcinosis observed in the animals fed the phosphate-supplemented commercial laboratory diet was almost certainly phosphate-induced, since no evidence of nephrocalcinosis was found in the animals fed the basal commercial laboratory diet. Apart from the severity, the pattern of development and the distribution of renal calcification was similar in the groups fed the phosphate-supplemented alpha protein diet, again suggesting that the additional dietary phosphate played a role in the calcification process. The distribution and characteristics of the nephrocalcinosis seen with the unsupplemented alpha protein diet were somewhat different. There was never the evidence of inflammation, interstitial fibrosis, foreign-body giant cell reaction, and calcification in the collecting ducts and the ascending thick limbs of Henle in the inner stripe of the outer medulla that was found in the kidneys of rats fed the phosphate-supplemented diets. This suggests that the nephrocalcinosis found with the unsupplemented alpha protein diet is not typically phosphate-induced.

Despite the differences in nephrocalcinosis between the animals fed the basal or unsupplemented alpha protein diet and the animals fed the phosphate-supplemented diets, there appeared to be similarities in the way the intraluminal deposits were formed. Ultrastructurally, the morphologic characteristics of the deposits were similar, and there was the same involvement of the tubular epithelial cells surrounding the deposits. Corresponding findings have been reported with nephrocalcinosis induced by other agents. Intraluminal deposits have been found following chloride depletion,17 with magnesium deficiency,18,19 and with a casein-based diet.20 Apatite-like crystals have been observed in association with the nephrocalcinosis induced by calcium gluconate,21 parathyroid hormone,21,22 vitamin D,23,24 and mag-
nesium deficiency. Therefore, it seems that the development and formation of some calci
erous deposits may, in some ways, be similar, irrespective of the inducing agent.

There were two ultrastructural findings that were observed only with the phosphate-supplemented diets. One of them was the electron-dense mem-
branes, which have not been previously reported by investigators studying nephrocalcinosis. It was presumed that the electron density of the membranes was due to impregnated calcium salts. It is highly unlikely, on the other hand, that the electron density was due to a staining artifact, inasmuch as grid staining was never used and electron-dense membranes were never found in control tissues, which were processed simultaneously with the experimental tissues. The other unique feature found was the laminated deposit, which was present in the region of the basal lamina of collecting ducts. Some other tubules may have been affected; however, severe tubular necrosis precluded their identification. Although laminated deposits have not been reported by anyone investigating nephrocalcinosis induced by dietary phosphate, similar structures have been reported in relation to renal basement membrane calcification induced by calcium gluconate, vitamin D, and intraperitoneal injections of phosphate. Because basement membrane calcification was not very obvious at the light-microscopic level, it is likely that its presence was masked by the prominent intraluminal deposits.

The biochemical estimations provided some interesting results. In the groups fed the phosphate-supplemented diets there was evidence of secondary hyperparathyroidism, particularly in the 3- and 6-week series. Serum and urinary phosphorus was elevated, and serum calcium was depressed. There was also a trend toward a decrease in the urinary excretion of calcium, although the results at 3 and 6 weeks were not significant. Results similar to these, in addition to morphologic evidence of parathyroid stimulation, have been reported in rats given phos-
phate by injection. Furthermore, La Flame and Jowsey have found an increase in circulating immunoactive parathyroid hormone in dogs given dietary phosphate supplements. Increases in the urinary excretion of cyclic AMP were also found in this study. It is currently accepted that the physio-
logic renal effects of parathyroid hormone are medi-
ated via an adenylate cyclase–cyclic AMP system. Some of the cyclic AMP produced is excreted. In addition, it has been shown that urinary cyclic AMP is derived almost exclusively from the kidney under the stimulation of parathyroid hormone. Further-

more, it has been demonstrated in man that parathyroid hormone is responsible for most of the cyclic AMP produced in the kidney that is contributed to the urine. In the present study, therefore, it is likely that with the phosphate-supplemented diets, there was stimulation of the parathyroid glands. This is significant because it seems that “phosphate-in-
duced” nephrocalcinosis is actually mediated through parathyroid hormone. Evidence for parathyroid stimulation in the animals fed the basal alpha protein diet is not present.

The overall conclusion of this study is that the nephrocalcinosis found in rats fed a diet containing 20% alpha protein is not typically “phosphate-in-
duced,” because the characteristics of calcification were different, at both the light- and electron-microscopical levels, from those found with phosphate-supplemented diets. In addition, there was evidence that the nephrocalcinosis found with the phosphate-supplemented diets may be mediated by parathyroid hormone, whereas with the unsupplemented alpha protein diet evidence for parathyroid hormone activity was lacking.

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